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Usefulness of concanavalin-A non-binding fraction of *Strongyloides venezuelensis* larvae to detect IgG and IgA in human strongyloidiasis☆,☆☆

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Abstract

Glycosylated components from *Strongyloides* have an important role in parasite establishment and host recognition of these substances. Considering the sugar-binding capacity of lectins, such as concanavalin-A (Con-A), IgG and IgA detection in serum samples from strongyloidiasis patients was tested using different antigenic preparations. The total saline extract (SE) of *Strongyloides venezuelensis* filariform larvae was fractionated in Con-A column to obtain Con-A unbound (Con-A UF) and Con-A bound (Con-A BF) fractions. Sensitivity (Se), specificity (Sp), area under the ROC curve (AUC), likelihood ratio (LR), and correlation coefficients were calculated. Con-A UF showed the highest diagnostic parameters for IgG detection (Se 95.0%, Sp 92.5%, AUC 0.99, LR 12.7) and high correlation ($r = 0.700$) with SE. Con-A fractions did not clearly demonstrate any usefulness for IgA detection. In conclusion, the results obtained demonstrate that Con-A UF is an important source of specific peptides efficient to detect IgG in strongyloidiasis immunodiagnosis.

Keywords: Strongyloidiasis; *Strongyloides stercoralis*; Glycoproteins; Serodiagnosis

1. Introduction

The *Strongyloides* genus includes 2 species of medical importance: *S. stercoralis* and *S. fuelleborni*. *S. stercoralis* has a cosmopolitan and heterogenous distribution, is endemic in the tropical and subtropical regions, and infects up to 100 million people (Grove, 1996; Olsen et al., 2009). Considering only active infections, strongyloidiasis is one of the major intestinal infections in humans; however, the number of people potentially exposed or with subclinical infection is much higher (Elliott et al., 2007; Siddiqui and Berk, 2001).

Clinical diagnosis is uncertain because most cases are oligosymptomatic or asymptomatic or present pulmonary and intestinal symptoms common to other parasitic diseases (Agrawal et al., 2009; Siddiqui and Berk, 2001). Due to uncertainty in clinical diagnosis, it is necessary to use parasitologic and immunologic tests. Since parasitologic tests rely on demonstration of larvae in stool specimens, problems in definitive diagnosis are related to cases with a minimal and irregular larval output (Hira et al., 2004; Liu and Weller, 1993). Considering the difficulties with parasitologic examination, detection of different classes of immunoglobulin anti-*Strongyloides* has importance in diagnosis as well as in host immune response evaluation (Atkins et al., 1999; Costa-Cruz et al., 1998; Mota-Ferreira et al., 2009; Rossi et al., 1993; Van Doorn et al., 2007).

Immunodiagnosis continues to be a challenge because of the difficulty in obtaining large amounts of *S. stercoralis* larvae for homologous antigen preparations. Alternative

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antigens using *Strongyloides cebus* (Campos et al., 1988), *Strongyloides ratti* (Costa-Cruz et al., 1998), and *Strongyloides venezuelensis* (Machado et al., 2003) have also been used with reasonable results using total antigenic preparations. Current serodiagnostic approaches in parasitology include fractionation techniques, proceeded by biochemical characterization and application in immunoassays (Feliciano et al., 2010; Gomes-Silva et al., 2008; Machado et al., 2007; Oliveira et al., 2010; Ribeiro et al., 2010).

Components from cuticle, amphids, and esophageal glands of *Strongyloides* showed an important role in parasite establishment and host recognition of these substances (Maruyama et al., 2000). Several of these components are glycosylated, and lectins, such as concanavalin A (Con-A) which binds especially in mannose residues, present affinity to these molecules (Maruyama and Nawa, 1997; Tobata-Kudo et al., 2005). Therefore, considering the sugar-binding capacity of lectins, the importance of *Strongyloides* glycoconjugate antigenic properties, and their potential in diagnosis, we aimed to evaluate Con-A unbound (Con-A UF) and bound fractions (Con-A BF) obtained from total saline extract (SE) from *S. venezuelensis* larvae in human strongyloidiasis serodiagnosis.

2. Materials and methods

2.1. Serum samples

The Research Ethics Committee for Human Research of the Universidade Federal de Uberlândia (UFU), Brazil, approved this study. Samples from 120 individuals were analyzed and divided into 3 groups. Group 1 (G1) consisted of 40 patients living in an endemic area and with confirmed parasitologic diagnosis of strongyloidiasis using the Moraes (1948) method, based on positive larval thermo-hydrotropism, and the method of Lutz (1919), a gravity sedimentation technique. Control groups comprised 2 groups: Group 2 (G2) consisted of 40 patients with other intestinal parasitic diseases including *Ascaris lumbricoides* ($n = 8$), *Enterobius vermicularis* ($n = 6$), hookworm ($n = 8$), *Schistosoma mansoni* ($n = 3$), taenids (*Hymenolepis nana* and *Taenia* sp.; $n = 3$), *Trichuris trichiura* ($n = 2$), *Giardia lamblia* ($n = 5$), and samples from co-infected patients [*A. lumbricoides* + hookworm (1), *A. lumbricoides* + *H. nana* (1), *A. lumbricoides* + *G. lamblia* (1) or *A. lumbricoides* + *T. trichiura* (2); $n = 5$]; all samples tested were negative for the presence of *S. stercoralis*; Group 3 (G3) consisted of 40 healthy subjects, based on their clinical status and with 3 negative fecal samples tests (Lutz, 1919; Moraes, 1948).

All patients from G2 and G3 were truly negative for the presence of *S. stercoralis* by the time of sample's collection.

2.2. Parasites

Third-stage larvae (L3) of *S. venezuelensis* were maintained in cultures of feces from experimentally infected rats at the Universidade Estadual de Campinas, São Paulo, Brazil. Briefly, feces were mixed with charcoal (1:1), moistened with water, spread in uniform layers on Petri dishes, and incubated (28 °C, 72 h). Infective larvae were concentrated using the method of Rugai et al. (1954). The L3 were washed 5 times in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.2) and stored at −20 °C in PBS.

2.3 Saline extract from S. venezuelensis

S. venezuelensis filariform larvae (250,000) were resuspended in PBS (0.01 mol/L, pH 7.2) containing protease inhibitors (ethylenediaminetetraacetic acid 1 mmol/L, benzamidine 1 mmol/L, phenyl methyl sulfonyl fluoride 1 mmol/L, aprotinin 1 μg/mL, and leupeptin 2 μg/mL) and disrupted in cycles of freezing (1 min, −196 °C) and thawing/sonication (5 min, 40 kHz, 37 °C) (Thornton, Inspec Eletrônica, São Paulo, Brazil). After an overnight incubation period at 4 °C under gentle shaking, the suspension was centrifuged (12,400 × *g*, 30 min, 4 °C) and the supernatant was analyzed for protein content according to Lowry et al. (1951) and stored at −20 °C until use.

2.4. Con-A affinity chromatography of total SE

Total SE was loaded for affinity chromatography on Con-A immobilized on cross-linked 4% beaded agarose (Sigma, St. Louis, CA, USA) to obtain Con-A UF and Con-A BF. The column was prepared according to the manufacturer's recommendation, with modifications. Briefly, the column was pre-washed with 5 column volumes of wash and stock solution (1 mol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, and 5 mmol/L $CaCl_2$), equilibrated with wash and stock solution diluted twice in water—equilibration buffer (EB, pH 6.5–7.5), and loaded with 2 mg of SE diluted in EB passed in a slow flow. After that, Con-A UF was collected and its complete removal was made by washing the column until EB was protein free. Con-A BF was eluted with α-D-mannopyranoside (500 mmol/L) (Sigma) in EB. After use, resin was regenerated as described in the datasheet. Fractions with peak absorbance at 280 nm were pooled, and protein concentration was determined (Lowry et al., 1951).

2.5. IgG and IgA enzyme-linked immunosorbent assay

Block titrations of reagents (antigen preparations, sera, and conjugate) were made to optimize enzyme-linked immunosorbent assay (ELISA) conditions. ELISA for IgG and IgA detection in serum samples was carried out according to Costa et al. (2003). In summary, polystyrene microplates (Interlab, São Paulo, Brazil) were coated with total SE or Con-A fractions at concentrations of 5 μg/mL in carbonate bicarbonate buffer (0.06 mol/L, pH 9.6), incubated overnight at 4 °C in a final volume of 50 μL/well, and washed 3 times, 5 min each time with PBS containing 0.05% Tween 20 (PBS-T). Afterwards, samples diluted in PBS-T were added as follows: serum diluted 1:160 for IgG and 1:40 for IgA detection and incubated for 45 min at 37 °C. After a washing cycle, enzyme conjugates diluted in PBS-T were

added: peroxidase-goat anti-human IgG, Fc specific (Sigma), diluted 1:2000 for IgG detection, and peroxidase-goat anti-human IgA, alpha chain specific (Sigma), diluted 1:1000 for IgA detection; then plates were incubated for 45 min at 37 °C. After another washing procedure, the assay was developed by adding the enzymatic substrate consisting of hydrogen peroxide and orthophenylenediamine in 0.1 mol/L citrate phosphate buffer (pH 5.5, 15 min) followed by 25 μL/well of H_2SO_4 (2N) to stop the reaction. Optical densities (OD) were determined at 492 nm in an ELISA reader (Titertek Plus, Flow Laboratories, USA).

2.6. Statistical analysis

Analyses were performed using the GraphPad software package version 5.0. Optimum point for each condition of ELISA reaction and cut-off points were established using a 2-graph receiver operating characteristic curve (TG-ROC) combined with a receiver operating characteristic (ROC) curve (Greiner et al., 1995). ELISA index was obtained by the ratio between OD and cut-off. Values of EI greater than the optimum point of reaction for each extract were considered positive.

IgG or IgA detection performance using each antigenic preparation tested was analyzed in terms of sensitivity (Se) and specificity (Sp). The determined indexes were calculated with previously described formulas (Youden, 1950). ROC curves were built to describe test indexes (Martinez et al., 2003). Area under the ROC curve (AUC), an index of diagnostic accuracy, was calculated and values close to 1 indicate an informative test and values close to 0.5 indicate an uninformative test (Hanley and McNeil, 1982). Likelihood ratio (LR), which indicates how likely patients with strongyloidiasis are going to have a specified test detection compared with patients not infected by the parasite, was estimated as Se/(1 − Sp).

Correlations between levels of different antigenic preparations in each antibody class tested with *S. stercoralis* in group 1 were determined using Spearman (r) or Spearman rank test (r_s). Probability (P) values of <0.05 were regarded as significant.

3. Results

3.1. IgG and IgA detection by ELISA

As shown in Fig. 1, all samples were tested by means of ELISA using total SE and antigenic fractions from *S. venezuelensis* obtained after affinity chromatography in Con-A column. Considering IgG and IgA, respectively, different patterns of recognition were observed. The mean of IgG positivity by the 3 preparations was 89.2% for G1 and 9.2% and 12.5% for G2 and G3, respectively. When detecting IgA anti-*Strongyloides* in G1, mean reactivity was 79.2%; in G2, a mean of 10% showed cross-reactivity with the different antigens tested, although considering IgG detection the positivity increased from 12.5% to 37.5% in G3.

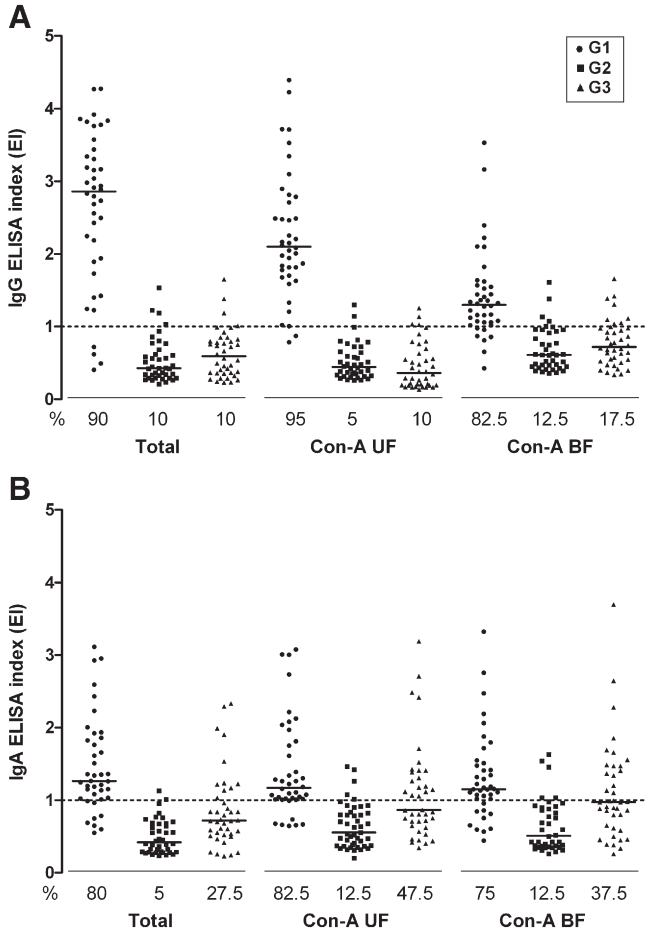

Fig. 1. Detection of IgG (A) and IgA (B) anti-*Strongyloides* antibodies in serum samples from patients with strongyloidiasis (G1; n = 40), other parasitic infections (G2; n = 40), and from apparently healthy individuals (G3; n = 40) by ELISA using total SE of *S. venezuelensis*, Con-A UF, and Con-A BF. The dotted line indicates ELISA index (EI); cut-off determined by ROC and TG-ROC curves.

The cross-reactivity in IgG ELISA in G2 was due to *Schistosoma mansoni* (1/3 ES and Con-A BF), *E. vermicularis* (1/3 ES and 2/3 Con-A BF), hookworm infection (1/8 ES, Con-A UF, and BF), and to co-infection (*A. lumbricoides* + *T. trichiura*; 1/5 ES, Con-A UF, and BF). Considering IgA detection, there were cross reactions with *T. trichiura* (1/2 Con-A BF), *A. lumbricoides* (1/8 ES and 2/8 Con-A UF and BF), and hookworm infection (1/8 ES, 3/8 Con-A UF, and 2/8 Con-A BF).

3.2. Diagnostic parameters

Coordinates of ROC curve and cut-off selection by TG-ROC presented a combination of Se and Sp as shown in Fig. 2. In agreement with the TG-ROC criteria established for SE in serum to detect IgG, the Se and Sp were both 90.0%, showing a great performance; Con-A UF for IgG recognition demonstrated an enhanced performance: Se of 95.0% and Sp of 92.5%. Overall, for IgA, the best indexes were achieved

when using SE (Sp of 83.8%) and Con-A UF (Se of 82.5%). Con-A BF produced poor parameters compared with the other 2 antigens either for IgG or for IgA detection.

Based on the evaluation of the ROC curve, Con-A UF is greatly efficient considering the discrimination between patients with strongyloidiasis and control groups, when measuring IgG. Test accuracy, indicated by AUC, showed that protocols for IgG and IgA detection with the different antigenic preparations are efficient and ranged from 0.77 to 0.99. Based on the AUC, values for IgG detection in serum samples using Con-A UF almost obtained a perfect value (1.00). Even with moderate efficiency, Con-A fractions triggered a considerable serologic cross-reactivity with G3 in IgA ELISA test, with indexes unexpectedly higher than SE; this behavior could partially explain the curve sketch (Fig. 2).

LR assessment conferred to IgG detection the best probability of a test result to be related with the existence of strongyloidiasis infection or effective immune response. For IgA, LR ranged from 2.9 to 5.0 (low to moderate values). There are no defined threshold scale results for LR, but when comparing SE and its Con-A fractions for IgG detection, Con-A UF showed a LR above 10, pointing out an exceptional information (LR 12.7).

Correlations and associations among serum levels of IgG or IgA anti-*Strongyloides*, SE, and Con-A fractions were analyzed in patients from G1 (Fig. 3). Patients with strongyloidiasis were found to have a positive correlation between IgG levels against SE and Con-A UF using the parametric Spearman coefficient ($r = 0.700$; $P < 0.0001$). It was also observed that there was a low correlation for IgG in Con-A UF versus Con-A BF ($r_s = 0.387$; $P = 0.014$). Moreover, a double-positive frequency in IgG detection of 90.0% for SE versus Con-A UF and 80% for Con A-BF versus Con-A UF was observed. Data from IgA showed moderate correlation coefficients, above 0.500, in 3

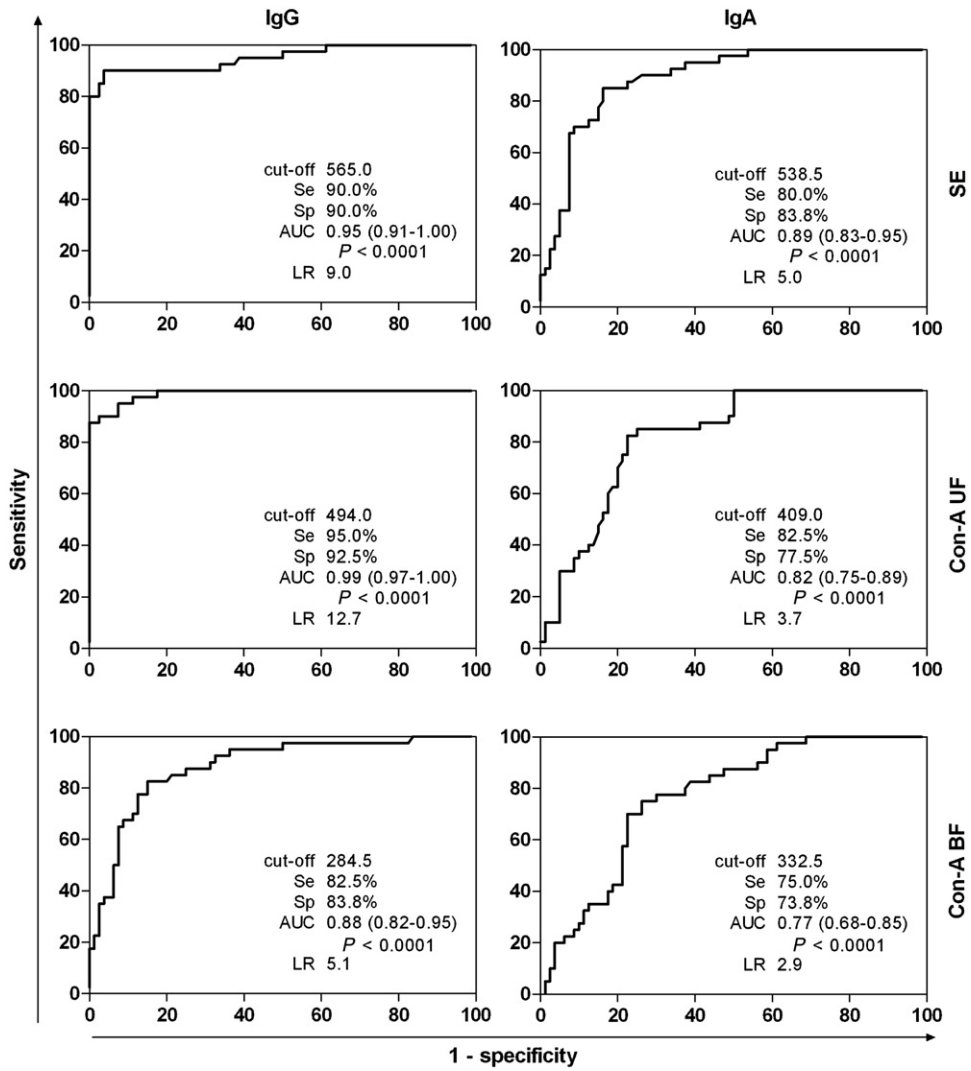

Fig. 2. ROC curve indicating the optimum point of reaction (cut-off), sensitivity (Se), specificity (Sp), AUC, and LR for total SE of *S. venezuelensis*, and Con-A UF and Con-A BF in serum samples for IgG and IgA detection.

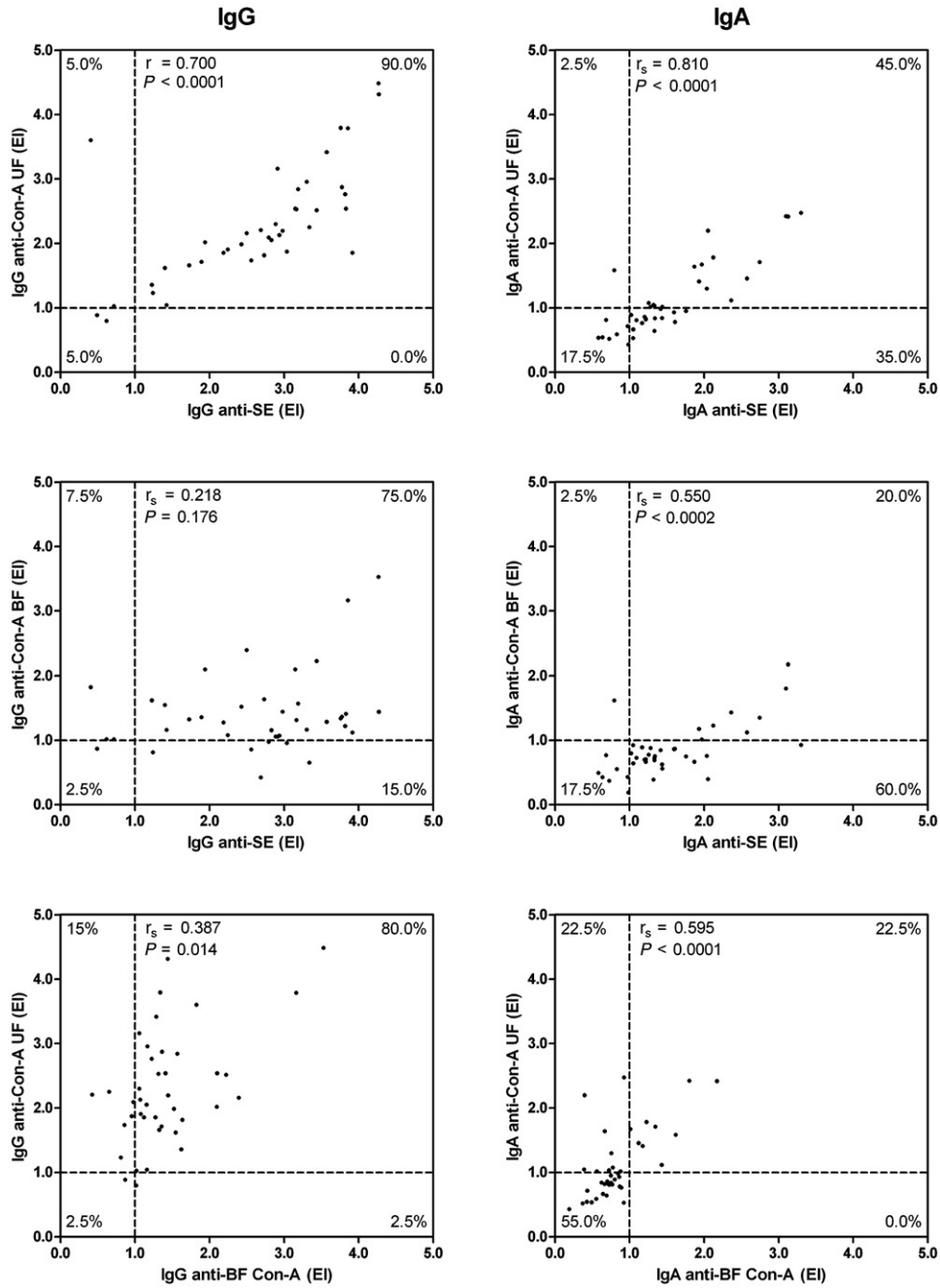

Fig. 3. Correlation and association between serum levels of IgG and IgA to total SE and concanavalin-A fractions—unbound (Con-A UF) and bound (Con-A BF)—from *S. venezuelensis* larvae in human strongyloidiasis patients (G1; $n = 40$). EI = ELISA index. Dotted lines indicate cut-off (EI = 1.0). Correlation coefficient of Pearson (r) and Spearman (r_s). Double or simple positive and negative results are indicated in the corresponding quadrants.

situations considering SE versus Con-A UF or Con-A BF (Fig. 3). A double-negative association was found for IgA when Con-A fractions were utilized (55%).

4. Discussion

Strongyloidiasis diagnosis is commonly supported by detection of specific antibodies (Agrawal et al., 2009; Siddiqui and Berk 2001). A limitation of serology is the high cross-reactivity indexes when using total antigenic extracts. Glycosylated epitopes in antigenic preparations are directly related to cross-reactivity in IgG immunodiagnostic tests for schistosomiasis (Alarcón de Noya et al., 2000), neurocysticercosis (Nunes et al., 2010), and leishmaniasis (Gomes-Silva et al., 2008).

Purification of *Strongyloides* larvae antigenic preparations already described includes the use of chromatographic techniques (Mangali et al., 1991; Rigo et al., 2008); however, this study is the first to employ the plant lectin

Con-A. Lectins have been used to obtain and evaluate glycosylated antigenic fractions in allergies (Alves et al., 2008), protozoan infections (Almeida et al., 1993; Gomes-Silva et al., 2008), and helminthiasis (Nunes et al., 2010; Oliveira et al., 2010; Rodriguez-Cannul et al., 1997) being a useful tool in serodiagnosis and to assess host immune response. Considering the outline in this study, the total SE of *S. venezuelensis* filariform larvae was fractionated by affinity chromatography in Con-A-agarose.

ELISA data demonstrated a different profile of IgG detection against the 3 antigenic fractions tested. Con-A UF presented an enhancement in Se and Sp, while Con-A BF showed the lowest diagnostic parameters. This indicates that different epitopes can be recognized and genus-specific IgG seems to bind less to glycosylated epitopes containing mannose residues. Additionally, a LR confirmed that Con-UF has an extraordinary performance to detect IgG in strongyloidiasis patients, and as proposed by Jaeschke et al. (1994), a LR value above 10 practically confirms disease diagnosis.

The significantly high correlation and association found in G1 between SE and Con-A UF for IgG detection indicate that this fraction contains major antigens involved in strongyloidiasis response, once 90.0% of G1 patients presented reactivity to both extracts. Even when not fully characterized, unbound fraction cross-reacted less with sera from patients with other parasitic diseases. The residual cross-reaction observed could be partially explained by the maintenance of reactivity with non–Con-A binding carbohydrates in glycoproteins or glycopeptides not fractionated.

IgG and IgA results showed different recognition patterns, because isotype responses are directed against distinct groups of antigens (Atkins et al., 1999; Genta et al., 1987). Fractions from *S. venezuelensis* saline antigen obtained by Con-A affinity chromatography did not clearly demonstrate the usefulness of IgA detection in strongyloidiasis patients with detectable larval output (G1). Correlation between SE and Con-A fractions in this group suggests that this may not be a source of specific peptides for IgA tests.

Some studies focused on evaluating IgA response in human strongyloidiasis (Atkins et al., 1999; Costa et al., 2003; Genta et al., 1987; Mota-Ferreira et al., 2009; Ribeiro et al., 2010; Rossi et al., 1993), but no one used purified antigens based on carbohydrate. Con-A fractions did not show high Se for IgA detection in strongyloidiasis patients. We hypothesize that purification exposed the different epitopes that cross-reacted with individuals who could have had previous contact with the parasite. A constant contact with antigenic components of *S. stercoralis* in the hyperendemic area where the study was conducted might also have led to an IgA response. Atkins et al. (1999) showed that IgA reacted with more antigens than the other isotypes tested and that reactivity with immunodominant components in Western blotting was significantly enhanced among coproNegatives. Differences in the percentage of *S. stercoralis* patients with detectable *Strongyloides*-specific IgA in other studies may be due to the heterogeneity of sampled patients (Rossi et al., 1993).

In conclusion, the results obtained herein demonstrate that antigenic fractions without affinity to Con-A, obtained from *S. venezuelensis* larvae, are an important source of antigenic compounds for detecting IgG in strongyloidiasis patients when tested by ELISA.

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